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VANIN-2, A POTENTIAL PROGNOSTIC MARKER OF RESISTANCE IN ACUTE LYMPHOBLASTIC LEUKEMIA

Candidature Dissertation to the Master's degree in Molecular Oncology submitted to the Institute of Biomedical Sciences Abel Salazar from University of Porto.

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Sumário

A Leucemia Linfoblástica Aguda (LLA) é o tumor pediátrico mais comum. Trata-se de uma doença genética complexa que resulta numa proliferação anormal e não controlada de precursores linfóides que impedem a diferenciação linfóide normal. Os progressos a nível do tratamento, devido ao avanço do conhecimento molecular da doença, levaram a uma taxa de sobrevivência de 5 anos superior a 85% nos países desenvolvidos. Contudo, a recidiva e a resistência à terapia ainda permanecem um desafio. Desta forma, a identificação de novas estratégias terapêuticas é necessária para não só para aumentar a sobrevida global mas também para diminuir a taxa de recidiva desta doença.

A proteína ancorada de superfície glycosylphosphatidylinositol Vanin-2, também conhecida como VNN2 e GPI-80, tem sido implicada na adesão e migração leucocitária e, mais recentemente, na função das células *stem* hematopoiéticas fetais. Anteriormente, o nosso grupo identificou a sobre-expressão da proteína VNN2 em doentes com risco muito elevado de recidiva bem como em doentes portadores do rearranjo *TCF3-HLF* resultante da translocação t(17;19)(q21-q22;p13), um subtipo raro de LLA associado a um mau prognóstico. Com o objetivo de explorar a relevância de VNN2 como um biomarcador, o nosso grupo demonstrou que a deteção de VNN2 na superfície membranar está associada com recidiva num estudo de coorte retrospetivo de doentes tratados com o mesmo protocolo clínico internacional. Para além disso, a VNN2 foi sempre detetada nos casos positivos para o rearranjo *TCF3-HLF*, o que parece justificar a inclusão prospetiva deste marcador na imunofenotipagem da LLA.

Para explorar a função da VNN2, nós estabelecemos uma abordagem de deleção da VNN2 através da libertação de um sistema lentiviral de CRISPR-CAS9 em células de LLA. Com este trabalho, demonstramos que uma redução eficiente da expressão desta proteína é atingida na superfície celular, o que fornece um sistema para futura validação funcional. No sentido de estabelecer um protocolo para deteção de VNN2 por citometria de fluxo procedemos à comparação e validação de dois anticorpos monoclonais, fornecendo as ferramentas necessárias para uma avaliação prospetiva da VNN2 como um biomarcador num estudo clínico cooperativo em curso. Para concluir, a descoberta da expressão aumentada da proteína VNN2 como um candidato a marcador de mau prognóstico na LLA pode contribuir para a melhor identificação da doença agressiva na LLA. O esforço desenvolvido nesta dissertação de mestrado irá contribuir para a avaliação tanto funcional como clínica da VNN2 na LLA.

Palavras-chave: Leucemia Linfoblástica Aguda, *TCF3-HLF*, recidiva, VNN2, anticorpo, CRISPR/CAS9.

Summary

Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy. ALL is a complex genetic disease that results in abnormal, uncontrolled proliferation of lymphoid precursors blocking normal lymphoid differentiation. The development of treatment due to molecular understanding improvement of the disease has led to a 5-year survival rate of more than 85% in developed countries. However, relapse and therapy-resistance still remain a challenge. Identification of novel therapeutic strategies is required to improve the outcome of refractory and relapsed ALL.

The glycosylphosphatidylinositol anchored surface protein Vanin-2, also known as VNN2 and GPI-80, has been implicated in leukocyte adherence and migration and most recently in fetal hematopoietic stem cell function. Previously, our group identified VNN2 as a unique feature in patients with very high risk of relapse by minimal residual disease as well as high surface levels in translocation t(17;19) (*TCF3-HLF*) positive patients, a rare subtype with a very poor outcome. To explore the relevance of VNN2 as a biomarker, we showed that detection of VNN2 at the surface of ALL was associated with relapse in a retrospective cohort of patients that were treated on the same international clinical protocol. Moreover, we found VNN2 to be always detected in *TCF3-HLF* ALL, which justifies prospective inclusion of the marker in ALL immunophenotyping.

To explore the function of VNN2, we have established a *VNN2* deletion approach by lentiviral delivery of CRISPR/CAS9 in ALL cells. We show that very efficient reduction of VNN2 expression in cell surface is achieved, which provides a system for further functional validation. We also contributed to standardize the protocol for detection of VNN2 by flow cytometry, comparing two monoclonal antibodies and validating them for this application. This provides tools for prospective evaluation of VNN2 as a biomarker in the setting of an ongoing cooperative clinical study. Taken together, the discovery of VNN2 as a candidate marker of worse outcome in ALL uncovers a so far unrecognized feature that is associated with aggressive disease in ALL. The endeavor developed in this Master Thesis will contribute to both functional and clinical evaluation of VNN2 in ALL.

Key-words: Acute Lymphoblastic Leukemia, relapse, VNN2, *TCF3-HLF* positive ALL, VNN2 antibody, CRISPR/Cas9 technique.

Abbreviation List

ALL – Acute Lymphoblastic Leukemia;

Cas9 – CRISPR associated protein 9;

cDNA – complementary DNA;

CNS – Central Nervous System;

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats;

DOX – Doxycycline;

FACS – Fluorescence-Activated Cell Sorting;

FMO – Fluorescence Minus One;

GFP – Green Fluorescent Protein;

HR – High risk of relapse;

HSC – Hematopoietic Stem Cell;

IR – Intermediate risk of relapse;

ITGAM – Integrin alpha M, also known as Mac-1;

LLA – Leucemia Linfoblástica Aguda;

MFI – Mean Flourescence Intensity;

MRD – Minimal Residual Disease;

NGS – NOD/SCID/IL2rγnull mouse;

PE – Phycoerythrin;

sgRNA – single guide RNA;

SR – Standard risk of relapse;

***TCF3-HLF* –** fusion gene t(17;19)(q21-q22;p13);

***TCF3-PBX1* –** fusion gene t(1;19)(q23;p13);

VHR – Very high risk of relapse;

VNN2 – Human glycosylphosphatidyl-inositol anchored cell-surface protein; also known as Vanin-2 or GPI-80.

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1. Introduction

1.1 Acute Lymphoblastic Leukemia

Leukemia, from Greek *leukós* (“white”) and *haĩma* (“blood”), is a malignant disorder of the hematopoietic system characterized by uncontrolled, neoplastic proliferation of leukocytes and their precursors. Leukemia can be classified as acute or chronic: whereas the acute form progresses very fast and is more common in children, the chronic one tends to grow slower and is typically present in adults. In another hand, it is possible to classify it between myeloid or lymphoblastic depending on the origin of the disease and the type of leukocytes affected. In this way, leukemia is divided in four common types: acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocitic leukemia (CLL) and chronic myeloid leukemia (CML).[1] The present thesis will focus on pediatric acute lymphoblastic leukemia.

1.1.1 Epidemiology

In the north part of Portugal, between 1997 and 2006, leukemia was the first common cancer identified in childhood (27%) followed by tumors of the central nervous system (CNS; 22%) and lymphomas (14%).[2] In 2012, leukemia still was the top one pediatric cancer in Portugal and worldwide.[3] Steady progress in development of effective treatments has led to a 5-year survival rate of more than 85% in developed countries[4, 5]. However, 25% of pediatric ALL patients still relapse.[5, 6] The precise assessment of the risk of relapse in individual patients is essential to ensure an intensive treatment primarily limited to high-risk cases and preventing the low-risk cases from unnecessary toxicities. To increase the survival rate and the quality of patients’ life is also required a better understanding of ALL pathobiology and the mechanisms of resistance for the development of new promising therapeutic strategies.[4, 6]

1.1.2 Pathogenesis

ALL is a genetic disorder malignancy of lymphoid progenitor cells which are supposed to differentiate into functional T or B-cells. These genetic disorders include mutations that lead to developmental arrest and unlimited self-renew.[7] The initial genetic lesions usually could be observed in utero and it requires additional submicroscopic genetic alterations to give rise to a full leukemia.[8]

In most of the cases, genetic lesions of diagnosis and relapse are different (Figure 1). Relapse often arises from the emergence of a minor subclone with genetic alterations distinct from those of the predominant clone at diagnosis. Since normally the relapse clone is present in low proportions at diagnosis and shares some lesions with the predominant one, these genetic alterations seem to confer resistance to the treatment.[8]

Thus, it is important to identify which exposures and inherited genetic variants are relevant and how and when they play a role in the complex multistep development of the acute lymphoblastic leukemia.

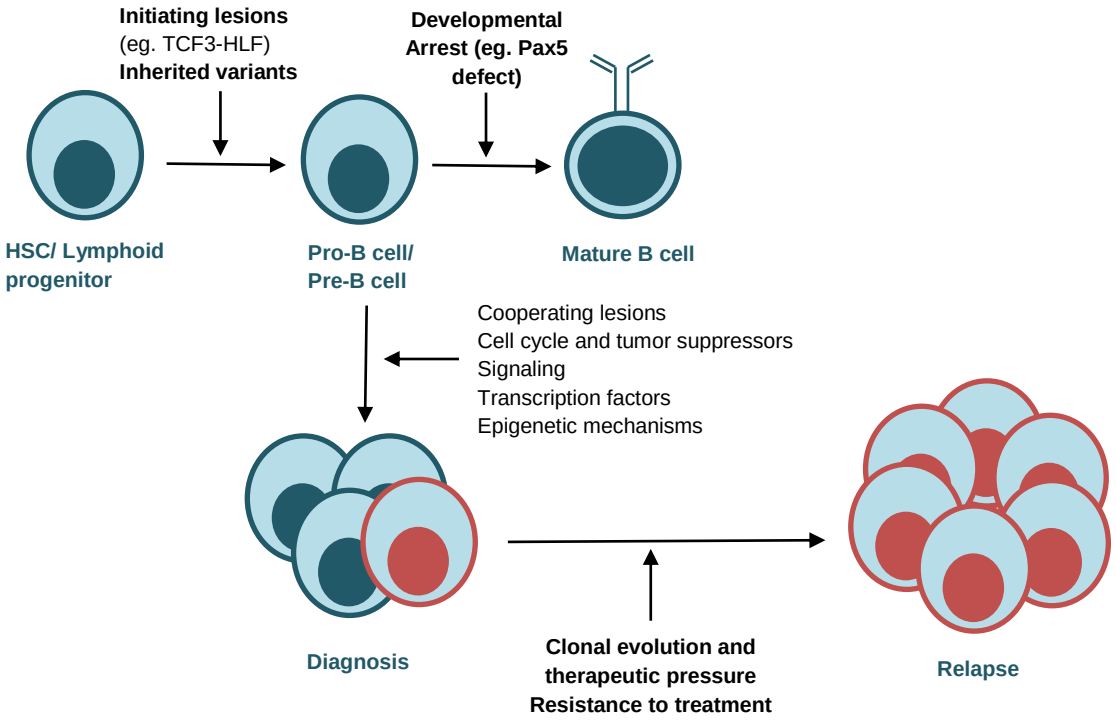


Figure 1 – Development of B lymphoblastic leukemia. The genetic alterations that contribute to leukemogenesis and the difference between diagnosis and relapse (adapted from Inaba et al, 2013 [8]).

1.1.3 Diagnosis

Patients suffering from ALL usually show common symptoms as general fatigue, fever, infections, unusual bruising, bone pain and enlarged lymph nodes, liver and spleen as a result of the impaired hematopoiesis in the bone marrow, lymphoid organs and extramedullary sites.[9]

For diagnosis, a bone marrow aspiration is always performed for multiple analyses. On one hand, leukemic lymphoblasts are examined by microscopy to see their morphology and identify the phenotype of leukemia. Moreover, flow cytometry is performed to measure the expression of specific leukemia-associated surface markers and, consequently, classify the lineage developmental and maturation stage based on the T-cell, mature B-cell and B-cell precursor phenotypes.[7] On the other hand, the genotype of the leukemia is assessed by chromosomal analysis, RT-PCR, fluorescence *in-situ* hybridization and flow cytometry thus leading to the identification of specific translocations, gain or loss of cellular DNA content and submicroscopic chromosomal abnormalities with prognostic or therapeutic relevance.[7, 8]

1.1.4 Treatment

The treatment for ALL typically consists of three different phases: remission-induction phase, intensification (consolidation) phase and continuation therapy.[7, 8] It normally takes 2 years and the dosages and schedule of chemotherapy are determined based on leukemic-cell biological features, response to treatment and pharmacodynamic and pharmacogenomic features of patients.[8]

The remission-induction phase (4-6 weeks) aims to eradicate the initial leukemic cell burden and to restore the normal hematopoiesis. The chemotherapy typically includes vincristine, a glucocorticoid (prednisone or dexamethasone) and a third drug (asparaginase, anthracycline or both). For standard-risk cases, after given an intensified post-remission treatment, this three-drug combination is sufficient to maintain the remission for most of the cases. However, for high-risk or very high-risk cases of ALL, additional drugs are needed. In pediatric ALL, clinical remission is achieved in 96-98% of the cases after this first phase of treatment.[7, 8]

After the remission-induction phase, normal hematopoiesis and body function are restored. Then, intensification (consolidation) therapy is given in order to eradicate drug-resistant residual leukemic cells and consequently the risk of relapse.[7] During this phase (2-4 months), methotrexate at high-dose in association with mercaptopurine is often administered as well as pulses of vincristine, glucocorticoids, uninterrupted asparaginase (during 20-30 weeks). Re-induction therapy can also be given when therapeutic fail occurs and drugs similar to those used in the first phase are administered.[7, 8] In addition, most of

the children with leukemia have subclinical CNS involvement at the time of diagnosis. Since leukemic cells are protected from systemic chemotherapy due to the blood brain barrier, which lead to 30-40% of the patients to relapse, CNS-directed therapy is a crucial requirement for successful ALL treatment.[1, 7]

Finally, in the last phase (continuation therapy) patients receive daily mercaptopurine and weekly methotrexate in two years or longer in order to maintain remission and prevent relapse. Dosage of the drugs is regularly adjusted according to the patient's leukocyte and neutrophil count.[7, 8]

For very high risk (VHR) patients or patients with poor initial response, allogenic hematopoietic stem-cell transplantation is required. In that case, the levels of disease burden should be reduced before transplantation. However, leukemia free survival is not dependent on the source of stem cells (matched related, matched unrelated, cord blood or haploidentical donor) but correlate with the minimal residual disease (MRD): high MRD levels are negatively associated with survival. However, even in patients that do not reach negative MRD, the ones that present low MRD levels (0,01-5%) can still benefit from the transplantation.[10]

The development and refinement of treatments have led to high cure rates of pediatric ALL of around 85%. Nevertheless, new treatments are still urgently needed for relapse cases. Besides, the effects to reduce the toxicity of chemotherapy are another research hot point. Identification of better prognostic markers and profiling with the employment of cutting-edge technologies, including next-generation genome sequencing and high-throughput drug screen, provides promising progresses of personalized treatments with more potent effect and less side effects.[4]

1.2 Prognostic factors in acute lymphoblastic leukemia

Careful risk stratification is essential to adjust chemotherapeutic treatment and to increase the patient survival rate. Clinical factors, genetic aberrations and treatment response are evaluated in order to estimate the prognosis (Table 1).

1.2.1 Clinical factors

The clinically relevant prognostic factors include age, leukocyte count, sex and immunophenotype. Children aged 1-9 have a better outcome than infants or adolescents, thus presenting a higher survival probability. Leucocyte count equal or higher than 50 x 10^9/L as well as male sex and T-cell immunophenotype are adverse factors that confer poor outcome.[8]

1.2.2 Biological factors – molecular genetics of ALL

Around 75% of pediatric ALL cases harbor one or more gross chromosomal alterations (Figure 2) and therefore cytogenetic characterization is commonly performed at diagnosis. However, only some genetic alterations are clinically validated as strong prognostic factors. Favorable outcome includes hyperdiploidy (with >50 chromosomes), and two translocations in B-progenitor ALL t(12;21)(p13;q22) encoding *ETV6-RUNX1* and t(1;19)(q23;p13.3) encoding *TCF3-PBX1*. In the other hand, hypodiploidy (<44 chromosomes), translocations in B-progenitor ALL (t(9;22)(q34;q11.2) and t(17;19) encoding *BCR-ABL* (Philadelphia chromosome) and *TCF3-HLF* respectively, immunoglobulin heavy chain locus (IGH) and mixed lineage leukemia gene (MLL) rearrangements are associated with poor outcome.[11, 12]

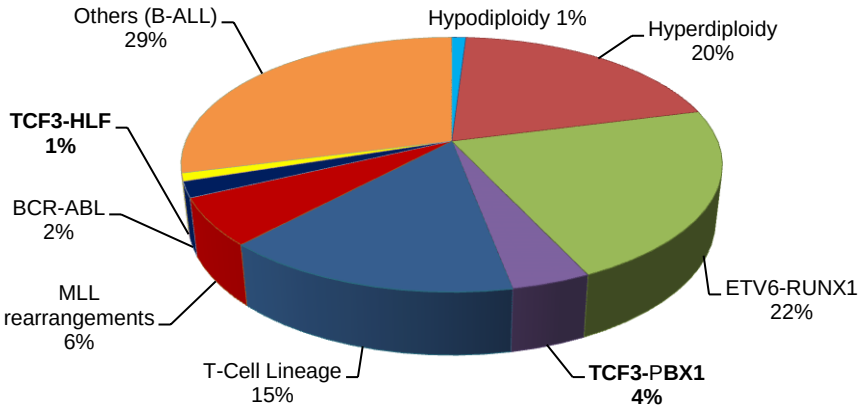


Figure 2 – Genetic alterations of pediatric ALL. The risk stratification is based on these genetic aberrations (adapted from Mullighan, 2013 [11]).

1.2.3 *In vivo* treatment response

Each patient has a different blast cell reduction in the peripheral blood (PB) or bone marrow (BM) after exposure to several anti-leukemic agents. Basically, the less time that is needed for reduction, the more sensitive is the patient to that treatment. The detection of MRD has considered to be the most powerful diagnostic tool in the contemporary ALL treatments since it allows monitoring *in vivo* response to treatment.[13]

Different informative checkpoints have been established in the treatment protocols and predefined MRD levels at these checkpoints can predict the risk of relapse. Patients with negative MRD (minimum sensitivity of 10^{-4}) on treatment day 33 (TP1, after remission induction therapy) and day 78 (TP2, after consolidation therapy) are considered standard risk (SR). Patients with positive MRD detection at either one or both time points but with levels $<10^{-3}$ at TP2 are classified as intermediate risk (IR). Finally, when high levels of MRD ($\geq 10^{-3}$) are detected in the TP2, patients are stratified as high risk (HR).[14]

The MRD levels can be assessed by two different techniques: flow cytometry analysis of the aberrant expression of leukemia specific antigens or quantitative RT-PCR detection of leukemia specific immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements or fusion transcripts. However, the sensitivity, applicability, time and cost are different for these techniques.[13]

	Favorable outcome	Unfavorable outcome
Clinical factors	• Age: 1-9 years old • Leukocyte count < 5 x 10^9/L • B-ALL	• Age: < 1 or > 10 years old • Leukocyte count > 50 x 10^9/L • T-ALL
Cytogenetic abnormalities	• High hyperdiploidy (51-65 chromosomes) • t(12;21) encoding *ETV6-RUNX1* • t(1;19) encoding *TCF3-PBX1*	• Hypodiploidy (less than 44 chromosomes) • t(17;19) encoding *TCF3-HLF* • t(9;22) encoding *BCR-ABL* (Philadelphia chromosome) • MLL gene rearrangement at 11q23 • IGH translocations
Minimal residual disease	• Standard risk patients: negative MRD ($\leq 10^{-4}$) at day 33 and 78 of treatment	• Intermediate risk patients: MRD level $<10^{-3}$ at day 78 of treatment • High risk patients: MRD level $\geq 10^{-3}$ at day 78 of treatment

Table 1 – Factors used for pediatric ALL risk stratification.

1.3 Characterization of the t(1;19) and t(17;19) ALL translocations

TCF3 gene (also termed E2A in the past) encodes transcription factors (E12, E47 and E2-5) that function as specific regulatory elements in target-gene promoter or enhancer regions and are essential for normal lymphopoeisis, especially for B-cell development.[15]

Chromosomal translocations generally create a fusion gene that encodes for a chimeric protein with novel structural and functional properties. TCF3 proteins can be converted into oncogenic transcription factors and two distinct translocations associated with TCF3 are found in pediatric ALL: t(1;19)(q23;p13) and t(17;19)(q21-q22;p13) that encode for *TCF3-PBX1* and *TCF3-HLF* respectively (Figure 3). The t(1;19) translocation generates a chimeric transcription factor which results from the fusion of the N-terminal transactivation domains of TCF3 with the C-terminal and homeodomain of PBX1. In another hand, the fusion protein that results from the t(17;19) translocation contains also the N-terminal transactivation domains of TCF3 but it is fused with the C-terminal and the basic leucine zipper (bZIP) regions of HLF, a member of the PAR family transcription factors.[15, 16]

Since both translocations are derived from the translocation involving TCF3 but associate with opposite clinical outcome (*TCF3-PBX1* is associated with good outcome and the *TCF3-HLF* is not curable at the moment), they represent a good model to study the pathogenesis of TCF3 translocation associated ALL.

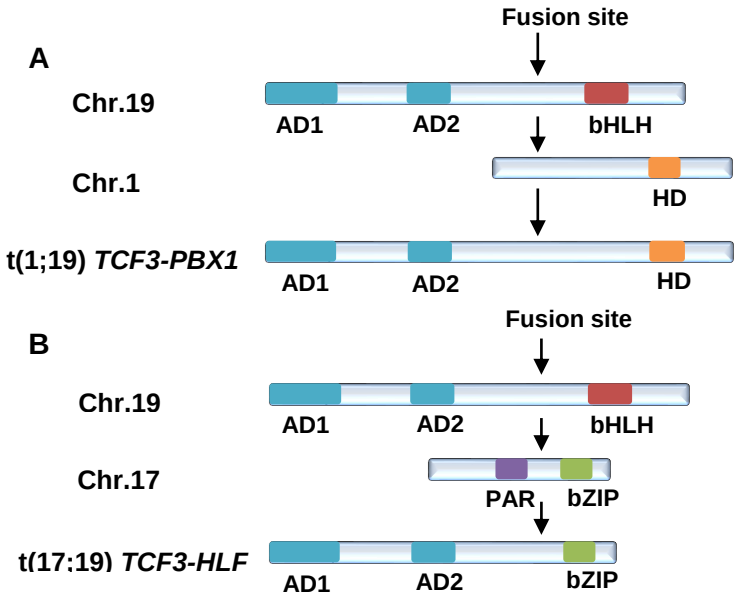


Figure 3 – Two TCF3 translocations in ALL that originate chimeric proteins.

(A) t(1;19)(q23;p13) encodes *TCF3-PBX1* and (B) t(17;19)(q21-22;p13) encodes *TCF3-HLF.* AD1/AD2 – transcriptional activation domains; bHLH – basic helix loop helix; HD – homeodomain;PAR – proline and acidic amino acid rich domain; bZIP – basic leucine zipper (adapted from Hunger, 1996 [15])

1.4 VNN2

1.4.1 Characterization of VNN2 and its function

Vanin-2 (VNN2), also known as GPI-80, is a human glycosylphosphatidylinositol (GPI)-anchored cell-surface protein. It is a member of the vanin (vascular non inflammatory molecule) family which expression has been demonstrated in colon, spleen, placenta, lung and leukocytes.[17]

The function of VNN2 is not well-characterized widely. In neutrophils, VNN2 is expressed on the surface membrane of the cells and is intracellular stored in secretory vesicles as a soluble form (without GPI-anchor).[18] VNN2 is involved in the neutrophil adherence and transendothelial migration processes.[19] It seems that it has a physical interaction with β2-integrin Mac-1 [20]. When adhesion to endothelial cells happens, VNN2 may dissociate from the Mac-1 and move to the pseudopodia of the neutrophil. Conversely, the chemotaxis leads to the release of VNN2 that is stored in the internal vesicles.[17]

In a recent study, Prashad and colleagues proved the expression of VNN2 in the human fetal liver, defining a population of cells with hematopoietic stem progenitor cells property with self-renewal ability. These cells were able to migrate between fetal hematopoietic sites and the function relies on the colocalization of VNN2 and ITGAM on the cell surface.[21]

1.4.2 VNN2 clinical significance in pediatric ALL

Previously, in our group, a cell surface glycoproteomics profile has been performed in ALL patients' samples in order to identify cell surface markers that may be characteristic for resistant disease. The surface proteomes of 19 B-cell-precursor-ALL (BCP-ALL) patient-derived xenografts including 8 very high risk patients and 11 standard risk patients with an excellent clinical outcome were defined in our previous studies using the Cell Surface Capturing (CSC) technology[25]. With this method, 13 proteins that were detected preferentially in VHR-ALL were selected (Figure 4), most of them being involved on cell adhesion and migration (VNN2, VNN1, ITGAM, CD33, CD302 and CEAM1). Specially, VNN2 was only found in VHR ALL patients (4 of 8) and ITGAM was co-expressed in 3 of them. The co-expression of these two genes has recently been described as important for self-renewal of human fetal hematopoietic stem cells, whereby RNA interference with VNN2 or ITGAM both impaired survival in vitro and engraftment/hematopoietic reconstitution ability in xenografts.[21] One of the 4 VNN2 positive patients was also a *TCF3-HLF* positive case, leading our group to focus on this protein.

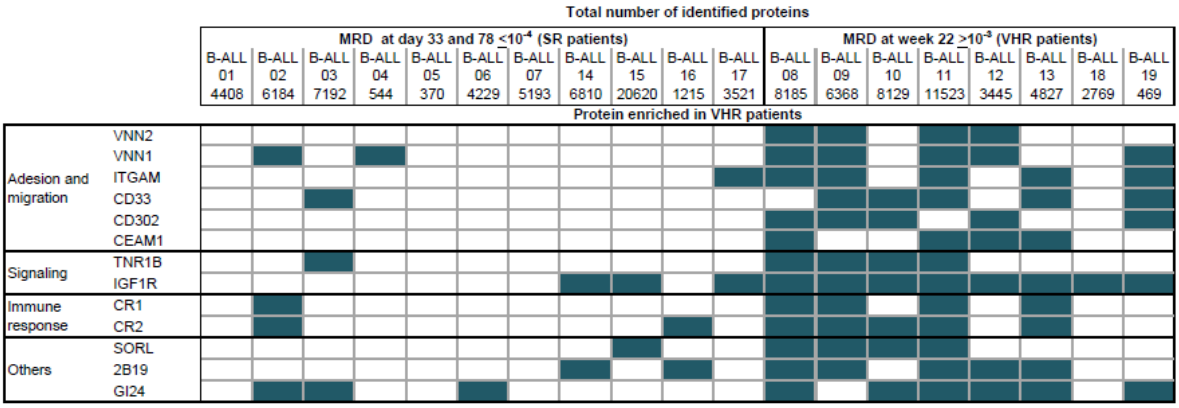


Figure 4 – Surface proteome of 19 BCP-ALL cases. 8 VHR and 11 SR BCP-ALL cases are compared.

On a second step, a cohort of 663 pediatric ALL patients from the clinical study ALL-BFM-200 was analyzed retrospectively by our group in order to prove that high VNN2 expression correlates with high risk disease and relapse. Quantitative PCR was performed in 209 SR-ALL, 345 IR-ALL and 109 HR-ALL cases (Figure 5). Very high levels of VNN2 transcripts were mostly identified in IR and HR ALL. Moreover, the expression levels were significantly higher in IR-ALL (1,076 ± 0.1522, p-value 0.0073) and HR (2,502 ± 0.6765, p-value 0,0001) patients compared to SR-ALL (0,5409 ± 0.0444). To further explore the relevance of this data, univariate Cox model was used and a threshold value for VNN2 expression was established which corresponds to a normalized delta CT value of 1.13 (Figure 5, dashed line). Using this threshold, 57% of HR-ALL, 27% of IR ALL and 12% of SR-ALL were defined as positive, suggesting that high VNN2 expression is associated with high risk disease.

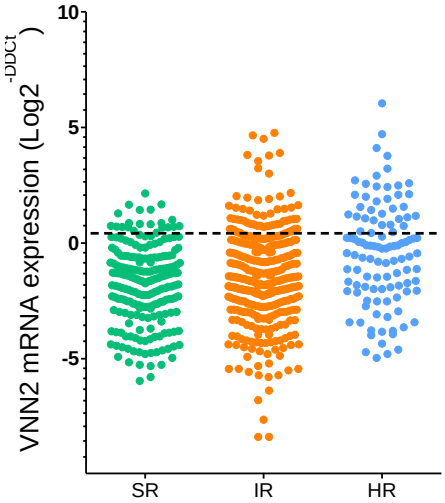
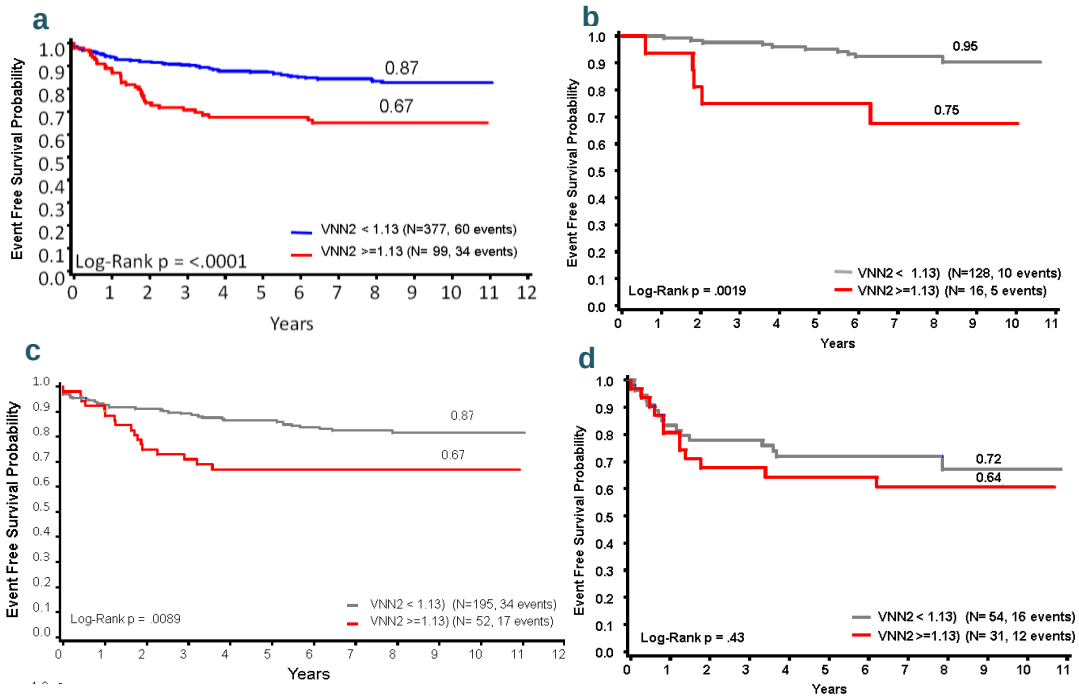


Figure 5 – VNN2 mRNA expression of 663 ALL patients. SR (n=209), IR (n=345) and HR (n=109). VNN2 mRNA is presented as logarithmic value of $2^{-\Delta\Delta Ct}$ (ΔCt=CtVNN2-CtSDHA) normalized for a reference sample with lowest VNN2 expression. The VNN2 expression threshold relative to this cohort is 1.13 (black dashed line).

The median event-free survival (EVS) probability of VNN2-high ALL was significantly lower than the rest of this cohort (Figure 6a). When comparing with each subgroup, significant effects were observed in IR-ALL (Figure 6b) and in SR group (Figure 6c) while in HR-ALL, VNN2-high cases did not further predict relapse than the remainder of the HR patients in this cohort (Figure 6d). Since the HR-ALL group receives more intensive post-induction chemotherapy, it is possible that treatment intensification may not provide a survival advantage in this subgroup of patients.



6 – Event Free Survival analysis (EFS) of the different risk groups based on VNN2 threshold (1.13) predictive of higher risk of relapse. EFS of VNN2-high ALL vs. EFS from rest of the cohort (a), EFS of VNN2-high vs. EFS from rest of the SR subgroup (b), EFS of VNN2-high vs. EFS from rest of the IR subgroup (c) and EFS of VNN2-high vs. EFS from rest of the HR subgroup (d).

Furthermore, another retrospective experiment was undertaken to explore if VNN2 detection by flow cytometry could provide a more strict identification of ALL cases at risk. Cases positively detected by flow cytometry and qRT-PCR for VNN2 where selected and their EFS was compared to the group of patients in the same cohort that was defined as VNN2 negative (Figure 7). A significant difference was observed as 50% of the VNN2 positive cases have relapsed.

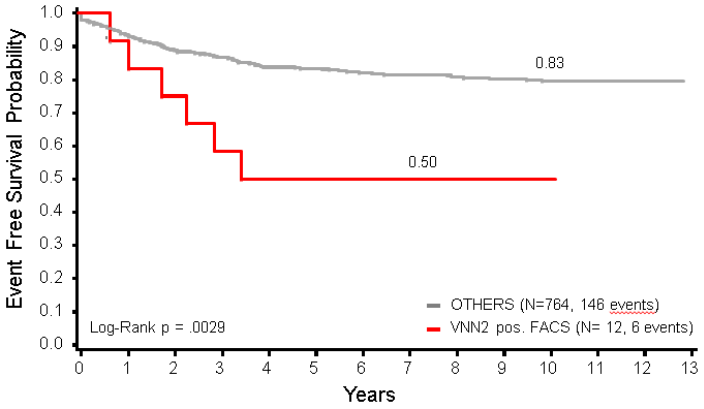


Figure 7 – Event Free Survival analysis (EFS) based on flow cytometry and qRT-PCR VNN2 expression assessment.

Surface expression of VNN2 could be a potential prognostic indicator that identifies *TCF3-HLF* positive ALL as well as a fraction of cases with high risk of relapse. Detecting its expression at diagnosis, could distinguish some of relapse cases that are currently considered as IR. With treatment adjustment, the survival probability of these cases could be improved.

2. Aims of the thesis

Regarding the fact that VNN2 is highly expressed in IR and HR ALL patients, especially on the *TCF3-HLF* cases, the principal aims of the present thesis were:

- **To investigate the functional role of VNN2 in pediatric ALL;**

Using the cutting-edge CRISPR/Cas9 technique to knockout *VNN2* in ALL cell lines and patients' samples to investigate if VNN2 play a role on the leukemia cells homing and engraftment.

- **To validate a VNN2 antibody for diagnosis;**

To compare the sensitivity and specificity of a new commercially available VNN2 antibody (clone 04) with another one (clone 3H9) that was described in most of the studies done up to date. For that, a cell line overexpressing VNN2 was generated and the antibody's performance was tested by FACS. The new antibody was also validated on patient-derived xenograft samples through the comparison between both with flow cytometry.

3. Materials and methods

3.1 Cell culture

Human Embryonic Kidney 293T cells (HEK 293T; DSMZ) were cultured in Dulbecco's modified eagle's medium (DMEM; Sigma® Life Science) supplemented with 10% Fetal Bovine Serum (FBS; Sigma® Life Science), L-Glutamine (2 mM; AMIMED) and Penicillin Streptomycin (P/S; 100 IU/ml; Gibco® by Life Technologies). The cells were detached from the bottom before splitting using Trypsin-EDTA PBS 1:250 (AMIMED).

The cell lines 658 (E2A-HLF positive; generated by B. Marovca in our group by cultivating xenograft patient material (patient ID: L707)) and Nalm6 (t(5;12); DSMZ) were maintained in RPMI-1640 medium (Sigma® Life Science) supplemented with 10% heat-inactivated (h.i.) FBS, L-Glutamine (2mM) and P/S (100 IU/mL).

All cultured cells were kept in the incubator at 37°C, 5% CO_2. Cells were thawed by putting them for 1 minute into a 37°C water bath followed by gentle addition of RPMI-1640 medium 10% FBS. To freeze the cells, h.i. FBS with 10% dimethyl sulfoxide (DMSO, Sigma® Life Science) was used as freeze medium and subsequently the cells were stored at -80°C.

3.2 CRISPR/Cas9 nuclease RNA-guided knockout of *VNN2*

The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system is a recently emerged technique for genome editing adapted from type II prokaryotic CRISPR immune system. The use of a single-guide RNA (sgRNA) associated with Cas9 nuclease allows the introduction of DNA-double strand breaks (DSB) at specific genomic sites. The sgRNA contains 20 nucleotide guide sequence that can be designed to target a specific gene. The only requirement for the selection of Cas9 target sites is the presence of a protospacer-adjacent motif (PAM) immediately downstream of the target site (5'-NGG). Targeted cleavage of the genome by Cas9 may lead to loss-of-function mutations since sgRNA targets to coding regions of genes and, consequently, can create frame shift insertion/deletion (indel) mutations.[22] Simultaneous lentiviral delivery of Cas9 and sgRNA throughout a single vector enables application of this technique in almost any cell type of interest, without the need to first generate cell lines that express Cas9.[23]

3.2.1 Targeting sites selection

Genome sequence of *VNN2* (Gene database from NCBI) was analyzed by http://crispr.mit.edu to design a suitable targeting sequence for sgRNA. Then, the best sequence was chosen and used to create the forward primers. At the 5' prime site of the forward and reverse primer the sequences CACCG and AAAC were added respectively

for cloning into LentiCRISPR-GFP vector (from Feng Zhang Lab). Primers were ordered from Microsynth.

Name	Primers
***VNN2*-F1**	CACCGTACCAACCTGCAGGGTTATT
***VNN2*-R1**	AAACAATAACCCTGCAGGTTGGTAC
***VNN2*-F2**	CACCGACGGAATCCAGTTCACCTGA
***VNN2*-R2**	AAACTCAGGTGAACTGGATTCCGTC
***VNN2*-F3**	CACCGGTGGAGTCACGGGAATTACA
***VNN2*-R3**	AAACTGTAATTCCCGTGACTCCACC
***VNN2*-F4**	CACCGGTGACTTTCAACACCGCATT
***VNN2*-R4**	AAACAATGCGGTGTTGAAAGTCACC
***VNN2*-F5**	CACCGTTGGGTAGGCAAGCGAGGAT
***VNN2*-R5**	AAACATCCTCGCTTGCCTACCCAAC

Table 2 – Primers selected for lentiviral CRISPR targeted *VNN2* knockout.

3.2.2 Plasmid construction

Lyophilized oligo primers were diluted with H_2O to a final concentration of 100 µM. To anneal the single stranded primer pairs, 10 µL forward and reverse primers were mixed with 10 µL NEB Tango buffer (Thermo Scientific) and 70 µL H_2O per each primer pair. To anneal the oligo the samples were heated up to 95°C and kept for 5 minutes at this temperature. After the samples had reached room temperature, annealed double-stranded oligos were cloning into linearized lentiCRISPR-GFP plasmid by T4 DNA ligase (Thermo Scientific).

3.2.3 Transformation of chemical competent bacteria

5µL of ligation product from the step before was added to 50 µL chemical-competent bacteria E.coli DH5 alpha (Invitrogen). After 2 minutes on ice, the samples were heat shocked for 100 seconds at 42°C and then put back on ice. 200 µL of S.O.C medium (Sigma® Life Science) was added to each sample before incubation for 1 hour at 37°C with a minimum shake of 300 rpm. Subsequently, the bacterial suspensions were seeded on LB agar Amp^+ Petri dishes and incubated at 37°C overnight.

3.2.4 Colony PCR for identification of plasmid of interest

From each plate, three colonies were picked to check oligo insertion using PCR. The colonies were pricked with a 20µL pipetting tip and added in PCR solution (0,5µL U6 forward primer, 0,5µL specific oligo reverse primer, 5µL ReadyMix™ Taq PCR Reaction

Mix (Sigma-Aldrich®) and 4µL water. Next, the following program was run: 1 cycle of 3 minutes at 95°C, 25 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 10 seconds at 72°C and an additional cycle of 10 minutes at 72°C.

3.2.5 Amplification of transformed bacteria

One positive clone per each condition was chosen to amplify for DNA extraction. Colonies were picked by pricking them with a 20 µL pipetting tip and dropping the tip in a 15 mL Falcon tube filled with 5 mL of LB-medium Amp^+. The bacteria were incubated at 37°C overnight with shaking at 225 rpm (Certomat® BS-T, Sartorius).

3.2.6 Plasmid DNA extraction

DNA extraction was performed using QIAprep Spin Miniprep Kit (Qiagen). DNA concentrations were measured by Nanodrop® (ND-1000 Spectrophotometer, witec ag).

3.2.7 Virus production

For transfection, HEK 293T cells were plated at T75 cm^2 cell culture flask in 13 mL DMEM medium 10% FBS h.i., L-Glutamine (2 mM) and P/S (100 IU/ml) and let to reach 60 – 80% of confluence.

For second-generation packaging, the psPAX2 packaging vector (Addgene) was used. The transfection mix was prepared as follows: 30 µg transfer plasmid (plasmid of interest), 11,25 µg psPAX2 (packaging plasmid), 11,25 µg pVSV-G (capsid plasmid), 1,5 ml DMEM medium and 112,5 µl PEI “Max” (Cat-Nr:24765-2, Polysciences). After 5 seconds of vortexing and 20 minutes at room temperature, the entire mixture was added dropwise to the flask. After 4 hours at 37° C, 5% CO_2, the medium was aspirated and 13 ml of new DMEM medium completed with 10% FBS h.i., L-Glutamine (2 mM) and P/S (100 IU/ml) was added per plate. After additional 24 and 48 hours, the supernatant was collected and 13 ml of fresh medium added to the plate. Supernatants were passed through a 0.45 µm millipore filter (TPP®) and centrifuged for 3 hours at 25000g. Next, supernatants were discarded, virus ressuspended in 1mL of RPMI-1640 complete medium and were ready to do the transduction.

3.2.8 Nalm6 and 658 cell lines transduction

500’000 cells of each cell line were suspended in 500 µL virus and 0,5 µL polybrene (8ug/ul) in a 24 well-plate. After 24 and 48 hours, the medium was replaced by fresh medium and cells were considered free of virus.

3.3 pINDUCER lentiviral system for VNN2 overexpression

A third-generation inducible cDNA expression lentiviral system (pINDUCER21) from Stephen Elledge (Meerbrey et al Proc Natl Acad Sci U S A. 2011 Mar 1;108(9):3665-70)

was used in order to inducible overexpress VNN2. This system is a single-vector system where the transcription is activated after addition of doxycycline (dox).[24]

3.3.1 Primer selection for VNN2 overexpression plasmid construction

Nucleotide database from NCBI was used to see the coding sequence (CDS) of *VNN2*. The mRNA transcript variant 1 from homo sapiens *VNN2* was used. For the forward sequence, 25 nucleotides starting with the start codon were chosen. Immediately 5' to this sequence, 15 nucleotides homolog to the vector including a SpeI restriction site and an ACC sequence were added from cloning. The reverse sequence included 24 nucleotides with the stop codon and was followed at 3' by 15 nucleotides that were homologs to the vector and included the BstbI restriction enzyme. Primers were ordered from MicroSynth.

VNN2	**Primers**
Forward	**GCGGCCCCGA** **ACTAGT** **ACC** ATGGTCACTTCCTCTTTTCCAATCT Homolog sequence SpeI
Reverse	GATCATAGCTTTGCAAAATATTGTAATGTTATAG **TTCGAA** **TACCCATACGA** BstbI Homolog sequence

Table 3 – Primers selected for lentiviral pINDUCER21-VNN2 overexpression.

3.3.2 Plasmid construction

Lyophilized oligo primers were diluted with H_2O to a final concentration of 50 µM. The vector pcDNA3.1(+) (Invitrogen) containing the *VNN2* cDNA was used as a template (10 ng/µL) in the following PCR reaction: 0,5 µL forward and reverse primers were mixed with 10 µL 5x GC buffer, 1,5 µL DMSO, 1 µL dNTPs, 1 µL pcDNA3.1-VNN2, 0,5 µL Phusion® High-Fidelity DNA Polymerase (Life Technologies) and brought to a final volume of 50 µL with H_2O. Next, the following program was run: 1 cycle of 30 seconds at 98°C, 30 cycles of 10 seconds at 98°C, 30 seconds at 55°C and 2 minutes at 72°C and an additional cycle of 5 minutes at 72°C.

To obtain the PCR amplified VNN2, an agarose gel electrophoresis was run and the band corresponding to VNN2 was extracted using Gel Extraction Kit QIAquick (Qiagen). Subsequently, the PCR product was digested with BcuI and BstbI restriction enzymes (Thermo Scientific) at 37°C overnight: 2,5 µL from each restriction enzyme, 5 µL 10x NEB Tango buffer, 2,5 µg (maximum) of VNN2 cDNA and H_2O to a final volume of 50 µL. To clone VNN2 into the pINDUCER21 vector, a ligation reaction was prepared: 1 µL VNN2 cDNA, 1,5 µL linearized vector, 1 µL NEB T4 ligase buffer, 1 µL of T4 DNA ligase (0,5W/µL) and H_2O to a final volume of 10 µL. The sample was left for 1 hour at room temperature.

3.3.3 Transformation of chemical competent bacteria, amplification of transformed bacteria and plasmid DNA extraction

These 3 steps were done as described before. The exception is that here when we pick the colonies to amplify, we don't know which one is well constructed. It is only possible to confirm on a later step.

3.4.4 Digestion validation

Another digestion was done for each colony amplified in order to confirm the well construction of the plasmid. 2,5 µL SalI restriction enzyme (Thermo Scientific), 5 µL 10x NEB Tango buffer, 0,5 µg pINDUCER21-VNN2 were brought to a final volume of 50 µL with H_2O. The reaction was let to occur during 4 hours at 37°C.

3.4.5 Virus production

For transfection, HEK 293T cells were plated in 6 well-plate with 2 mL DMEM medium 10% FBS h.i., L-Glutamine (2 mM) and P/S (100 IU/ml) and let to reach 60 – 80% of confluence.

For second-generation packaging, the psPAX2 packaging vector was used. The transfection mix was prepared as follows: 4 µg transfer plasmid (plasmid of interest), 1,5 µg psPAX2 (packaging plasmid), 1,5 µg pVSV-G (capsid plasmid), 200 µL DMEM medium and 15 µl PEI. After 5 seconds of vortexing and 20 minutes at room temperature, the entire mixture was added dropwise to the well. After 4 hours at 37°C, 5% CO_2, the medium was aspirated and 2 ml of new DMEM medium completed with 10% FBS h.i., L-Glutamine (2 mM) and P/S (100 IU/ml) was added. After 48 hours, the supernatant was collected, centrifuged for 5 minutes at 650 rpm and was ready to do the transduction.

3.4.6 Nalm6 cell line transduction

Transduction was performed as described before.

3.5 Flow cytometry analysis and cell sorting

Flow cytometry analyses were performed using BD FACS Canto™ II (BD Biosciences).

BD FACS Aria™ III (BD Biosciences) was used for cell sorting.

3.6 658 *VNN2* knockout efficiency

658 transduced cells were rinsed with 1x PBS and incubated with 5 µg/mL of mouse IgG1 anti-human GPI-80 (clone 3H9, PE conjugated, MBL® International Corporation) for 20 minutes at 4ºC. After incubation, the cells were rinsed again with 1x PBS and VNN2 expression was measured by FACS.

3.7 Nalm6 VNN2 overexpression efficiency

Nalm6 transduced cells were treated with 0, 10, 100 or 1000 ng/mL doxycycline for 3 days. After dox treatment, cells were incubated with 5 µg/mL of mouse IgG1 anti-human GPI-80 (clone 04, PE conjugated, Sino Biological Inc.) for 20 minutes at 4ºC. After incubation, the cells were rinsed again with 1x PBS and VNN2 expression was measured by FACS.

3.8 VNN2 expression in patient-derived xenografts

Patient-derived xenograft samples were stained with mouse IgG1 anti-human-GPI-80 (PE conjugated, clones 3H9 and 04) using different concentrations of 1,25, 2,5, 5 and 10 µg/mL for 20 minutes at 4ºC. The same treatment procedure was applied for the isotype control (mouse IgG1, PE conjugated, abcam®). After incubation, the cells were rinsed with 1x PBS and measured by FACS.

3.9 Immunophenotyping

Following antibodies were used for immunophenotyping of xenoamplified human ALL cells: mouse anti-human CD10 (APC conjugate, Clone HI10a, Cat No 312210, BioLegend, Frederick, USA), mouse anti-human CD19 (PeCy7 conjugate, Clone SJ25C1, REF 25-0198-42, eBioscience Inc., San Diego, CA, USA), mouse anti-human CD14 (APCH7 conjugate), mouse anti-human CD34 (PerCpCy5 conjugate, REF 347222, BD Biosciences, San Jose, CA, USA), mouse anti-human CD2 (FITC conjugate) mouse anti-human CD45 (Pacific Orange conjugate, REF MHCD4530, Invitrogen, Frederick, USA). For VNN2 detection the mouse IgG1 anti-human GPI-80 antibody (PE conjugate, clone 3H9 or 04) was used. For compensation the following compensation beads were used: BD™ CompBeads Anti-Mouse Ig, κ (Cat No 51-90-9001229; BD Biosciences, San Jose, CA, USA).

The Mean Fluorescence Intensity change (Delta MFI) was calculated as the difference between MFI values of populations stained with the monoclonal antibody of interest and the fluorescence minus one control (FMO).

3.10 Computational analysis

Flow cytometry results were analyzed with the software FACSDiva (Becton Dickinson) and FlowJo (version 10, TreeStar).

4. Results

4.1 Overexpression of VNN2 by pINDUCER21 in ALL cells

To test the sensitivity and specificity of a new commercial available antibody for VNN2 (clone 04), a DOX-inducible VNN2 overexpression plasmid was transduced to nalm6 cells. After 3 days treatment of increasing concentration of DOX, the expression level of VNN2 on cell membrane was measured by flow cytometry after staining with PE conjugated VNN2 antibody (clone4) (Figure 8). As shown in the figure, without DOX treatment (0 ng/ml), transduction of pINDUCER21-VNN2 plasmid does not change VNN2 levels per se (from 1,61% to 3,89%). However, after DOX treatment, the transduced cells remarkably increased VNN2 expression level in a dose-dependent manner. Thus, the new VNN2 antibody (clone 04) was able to detect increase level of VNN2.

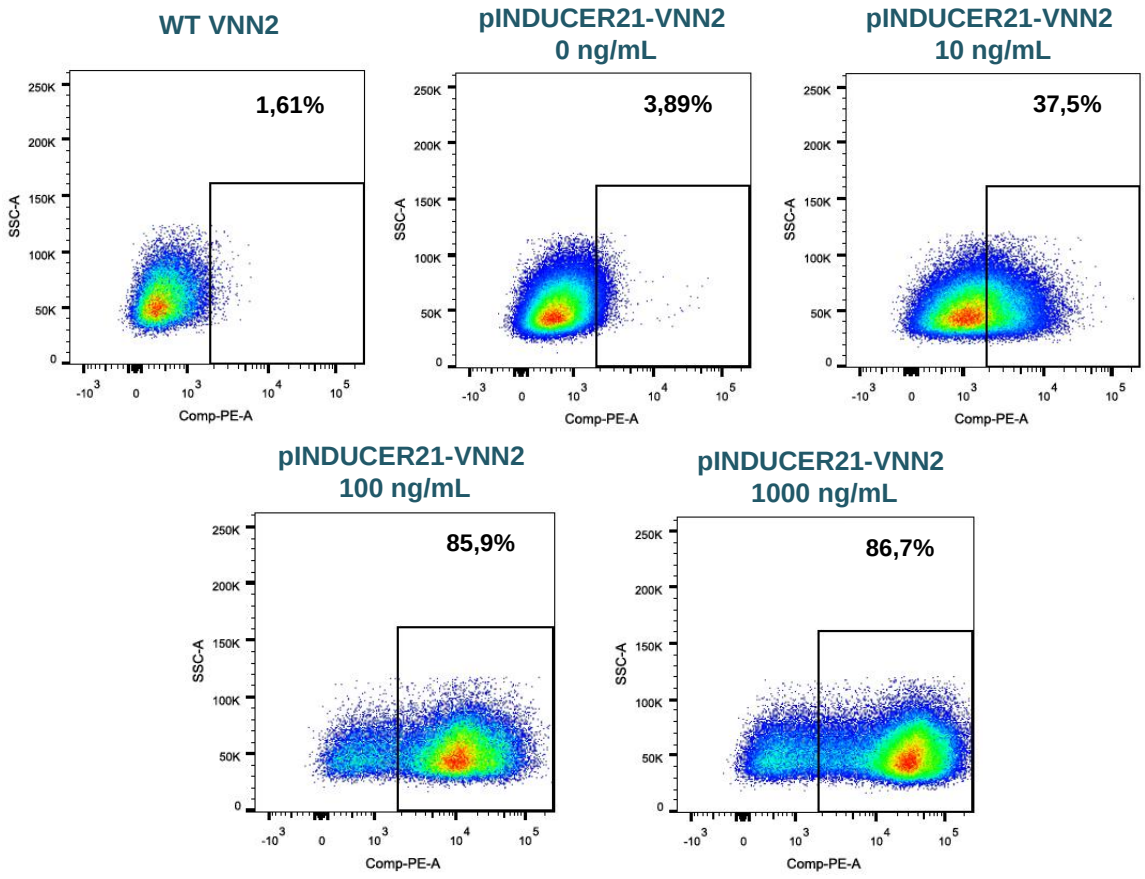


Figure 8 – pINDUCER21-VNN2 overexpression in nalm6 cell line. No transduced cells used as a control for gating the VNN2 basal levels. Increased dox concentrations were used to overexpress VNN2 in the transduced cells.

4.2 Validation of VNN2 expression in patient-derived xenografts

The VNN2 expression in two patient-derived xenograft samples, A11 (*TCF3-HLF* positive case) and G63 (*TCF3-PBX1* positive case), was measured by two commercial available antibodies (clones 3H9 and 04). Increasing concentrations of antibodies were used for this titration analysis (Figure 9). For A11, the signal intensity increased as increasing concentrations of antibody 04 were used. Antibody 3H9 reaches the saturation at concentration of 2,5 µg/mL in this case. However, at concentration of 10 µg/mL, the isotype control starts to show a considerable background suggesting that this concentration is too high to use. In the same setting, no VNN2 signal was detected in the G63 patient.

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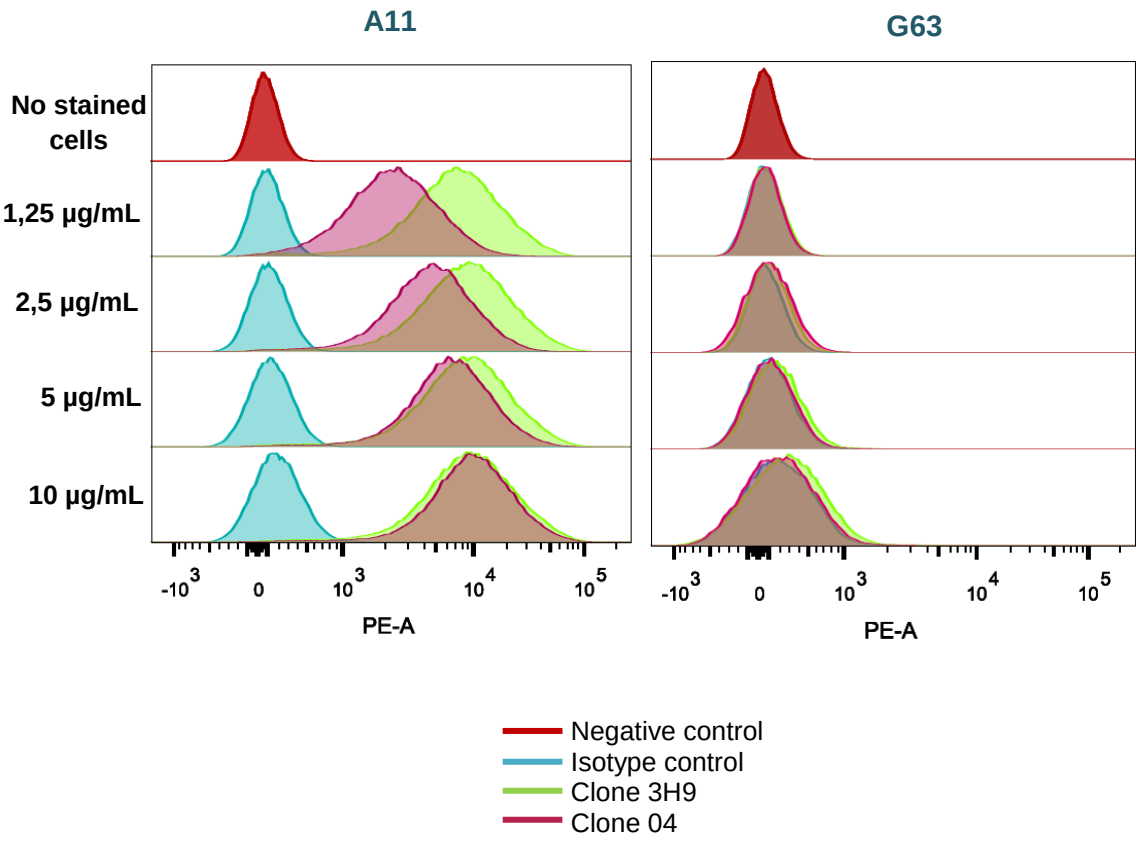

Figure 9 – VNN2 expression in a *TCF3-HLF* and *TCF3-PBX1* positive cases. VNN2 expression was validated with two anti-VNN2 antibodies (clone 3H9 and 04).

Next, the VNN2 expression in six *TCF3-HLF* positive ALL patients was tested by these two VNN2 antibodies at concentration of 5 µg/mL. As shown in figure 10, delta VNN2 MFI (VNN2 expression – FMO) for each antibody demonstrates that they detected VNN2 levels in all the patients. The signal intensity from 3H9 was higher than 04, suggesting 3H9 is more sensitive in this setting.

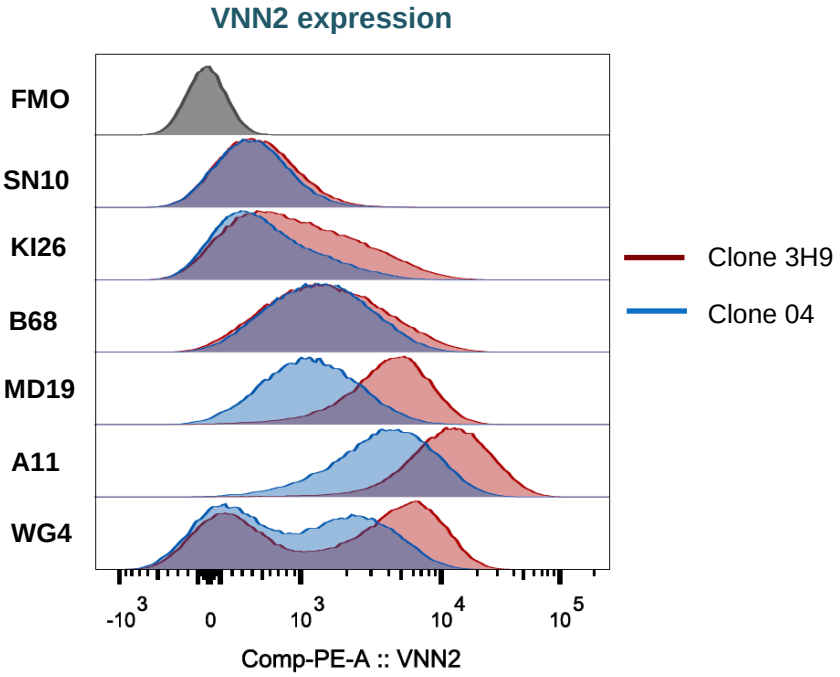

Figure 10 – VNN2 expression in 6 *TCF3-HLF* positive ALL patients. VNN2 expression was validated with two anti-VNN2 antibodies (clone 3H9 and 04).

4.3 Knockout of *VNN2* by CRISPR/Cas9 in ALL cells

Three weeks after transduction of *VNN2* knockout plasmid (CRISPR-VNN2), the cell membrane expression of VNN2 was measured by flow cytometry. After staining with PE conjugated VNN2 antibody, the VNN2 levels were compared between GFP^- (untransduced) and GFP^+ (transduced) populations. As shown in figure 11, the VNN2 levels are not different between GFP^- and GFP^+ population in cells transduced with two CRISPR-scramble plasmids (no specific-targeting in genome) suggesting transduction of CRISPR plasmid does not change VNN2 level per se. However, all 5 of the VNN2-targeting plasmid remarkably decrease the VNN2 level in transuded populations (GFP^+). Importantly, comparing the ratio between PE^- and PE^+ within the GFP^+ group, the sgRNA 2 and 5 generated the first two high ratio suggesting that these two sequences are more potent to knockout *VNN2* than the others. Thus, though the 658 cells only express moderate VNN2 on cell membrane, the remarkable decrease of VNN2 level in the cells transduced with CRISPR plasmid targeting *VNN2* suggests the knockout works in this cell line.

Another way to analyze the knockout efficiency is to gate the cell population in two groups (GFP^- and GFP^+) and compare the mean fluorescence intensity (MFI) of VNN2 of these two groups. As shown in Figure 12, the first panel indicate the basal level of VNN2 (blue curve) against background (red curve) in 658 cells. The next two panels represent the negative controls (scrambles 1 and 2): the VNN2 MFI from the GFP^+ population (pink curve) is quite similar to the one from the GFP^- population (green curve) confirming that the VNN2 level has no change in transduced population. The next five panels represent the five different sgRNAs that target *VNN2.* It is obvious that the VNN2 level is decreased in all the GFP^+ populations (pink curves). Again, within the five different constructions, the sequence number 2 is the most potent one.

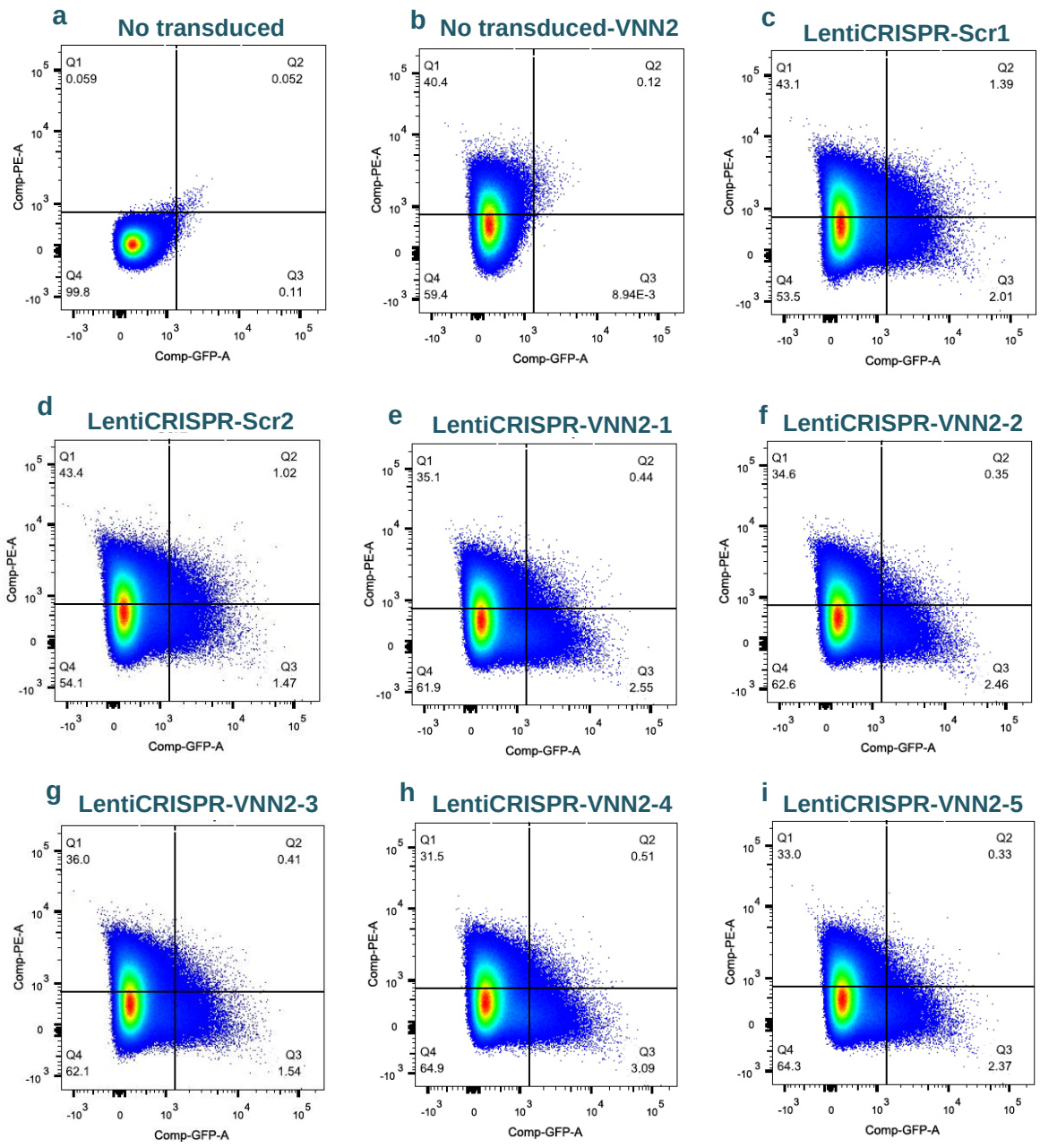

Figure 11 – LentiCRISPR-*VNN2* knockout in 658 cell line.

No transduced and no stained cells (a), no transduced and stained cells with PE-VNN2 antibody (b), lentiCRISPR scrambles 1 and 2 stained cells with PE-VNN2 antibody (c and d) and lentiCRISPR-VNN2 with five different targets cells stained with PE-VNN2 antibody (e, f, g, h and i).

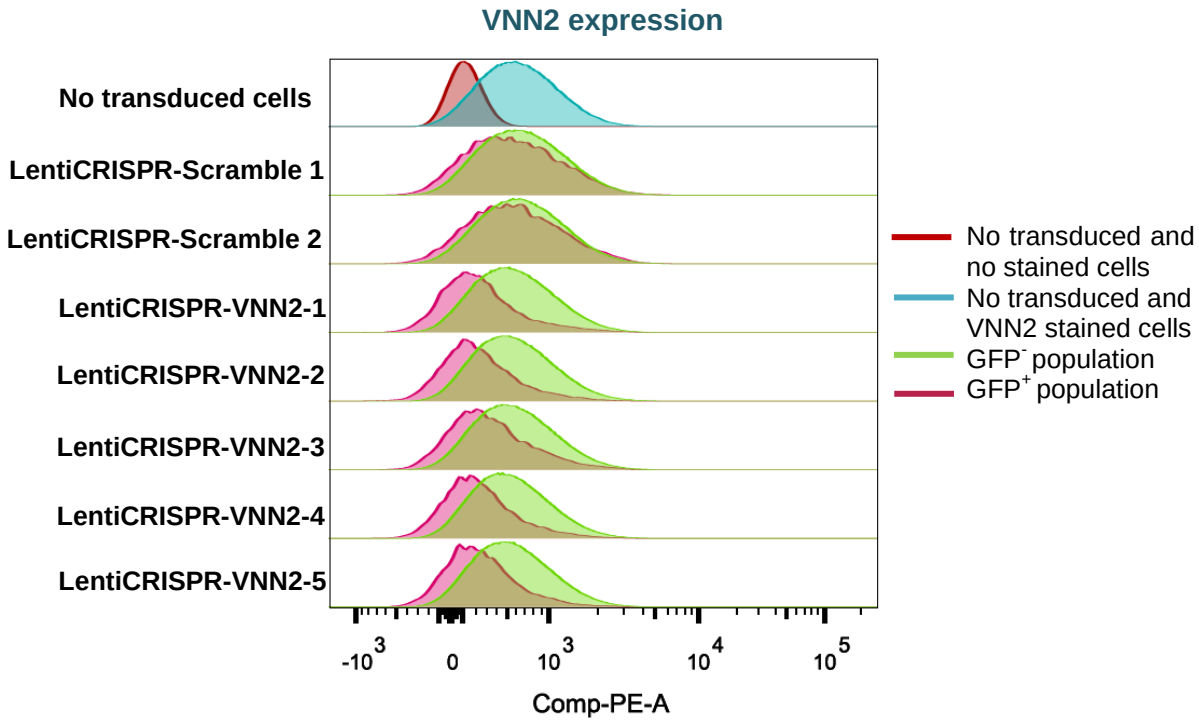

Figure 12 – LentiCRISPR-*VNN2* knockout in 658 cell line. No transduced and no stained (red curve) vs. no transduced and stained (blue curve) cells with PE-VNN2 antibody (3H9). GFP^- (green curve) vs. GFP^+ (pink curve) cells from transduced populations (lentiCRISPR) stained with PE-VNN2 antibody.

5. Discussion

5.1 VNN2 expression is associated with high risk disease and relapse in ALL

Through a surfaceome analysis of ALL patients belonging to different prognostic groups (SR, IR and HR), VNN2 was mostly identified in patients that are categorized as high levels of MRD disease after induction chemotherapy treatment (an indication of resistance to therapy). Within these, all the patients carrying the translocation t(17;19) (*TCF3-HLF*) have high levels of VNN2. The chimeric protein resulted from TCF3 and HLF gene rearrangement defines a subtype of ALL that is typically associated with relapse and poor outcome. Currently, this translocation is the only one that VNN2 is exclusively associated with. Thus, the detection of VNN2 by flow cytometry will provide a convenient and cost effective approach to select ALL cases for specific genetic testing of the *TCF3-HLF* translocation.

To further clarify this relationship between VNN2 expression and relapse, a retrospective analysis involving more than 600 pediatric ALL patients was performed. An enrichment of cases with high levels of VNN2 RNA expression was found in patients with intermediate risk and high risk of relapse. It was also possible to set a threshold of VNN2 expression level that distinguishes a subclass of patients with significantly higher risk of relapse. With this threshold, the EFS of VNN2-high cases from each group (SR, IR and HR) was compared with the EFS from the remaining patients from the respective group showing a tendency of relapse for that patients. Interestingly, the identification of patients in the IR group that predict relapse is of high interest since they could be subjected to more intensify treatments at the first place. This would change the fact that IR still constitutes the group with the largest number of relapses in ALL but for which currently no alternative markers are available. The detection of VNN2 by flow cytometry also demonstrated that 50% of patients with high levels of VNN2 will relapse. This data indicates that including of VNN2 in diagnostic flow procedures could contribute in identifying more potential high risk cases.

In this way, the risk stratification tools should include VNN2 surface expression as well as the genomic information and the MRD assessment in the future. These data provided the basis for an ongoing prospective evaluation of this biomarker on the international AIEOP-BFM-ALL-2009 trial including Switzerland, Germany, Italy and Austria. The inclusion of VNN2 in the diagnostic immunophenotyping panel of the study will serve to identify more effectively the *TCF3-HLF* positive cases that need alternative experimental therapies.

5.2 VNN2 antibody (clone 04) validation for diagnosis

In the present study, a new commercial available VNN2 antibody (clone 04) has been tested and compared with an old one that was being used before (clone 3H9). Clone 04 is produced from a recombinant human VNN2 while the 3H9 comes from human activated neutrophils. Neverthless, both of these antibodies recognize the peptide portion from the VNN2 surface membrane protein.

Through the establishment of a VNN2 inducible overexpression system and the use of different concentrations of dox to induce the protein expression, it is confirmed that the signal detected from the 04 antibody is regarded to VNN2. Thus, it makes it a rival of the 3H9. To clarify which one is more sensitive, both antibodies were tested at different concentrations on two patient-derived xenografts with opposite outcome (*TCF3-HLF* and *TCF3-PBX1*). With increasing concentration of both antibodies, it is evident that 3H9 reaches the saturation condition faster than clone 04. However, at concentration of 5 µg/mL, their behavior is similar. To further clarify this, 6 *TCF3-HLF* positive ALL cases were incubated with both antibodies at the same concentration (5 µg/mL). 3H9 presented more sensitive signal in 4 of the 6 patients. If each antibody recognizes different epitope from VNN2 and these are not equally distributed on the surface membrane from the cells, this could be an explanation for the differences observed. Nevertheless, 04 clone is also a good candidate.

5.3 A potential role of VNN2 on homing and engraftment of ALL

Explore the functional role of VNN2 expression in IR and HR ALL cases is one of the principle goals of this project. A *VNN2* knockout approach was established and validated in ALL cells using the lentiviral CRISPR/Cas9 strategy. To have a high chance to obtain an effect, five sgRNAs targeting *VNN2* (5 different exon of) were designed. All of them remarkably decrease VNN2 levels on a *TCF3-HLF* cell line (658). However, we are still far away to see a potential relationship as it was proposed. CRISPR is a cutting-edge genome-editing technique that can be employed to introduce a specific point mutation or correct a pre-existing mutation. The advantage of this system is that a completely eliminating gene function can be achieved, making it possible to study phenotypes that require a complete loss of gene function.[26, 27] Since VNN2 is involved on migration and adhesion of neutrophils[19], we expect a potential function of VNN2 on the engraftment and/or homing capacity of ALL cells. The establishment of a *TCF3-HLF VNN2* knockout cell line was the first step of this loss-of-function study. Confirmation studies are still required for these preliminary data. It is still needed to enrich or purify the knockout population by sorting the cells of interest and then test their efficiency in another way (eg. western blot). Alternatively, deep-sequence could be done to check and quantify the

genomic sequence edited by CRISPR/Cas9 system. After confirmed the knockouts, the next step is to delete *VNN2* in patient-derived samples and investigate the biological effect *in vivo*. The idea is to transplant *VNN2* knockout cells into NSG mice and track if *VNN2* knockout will change the behavior of these cells *in vivo* including homing and/or engraftment. Recently, Prashad and colleagues[21] have discovered that VNN2 distinguishes a population of human fetal liver hematopoietic stem cells (HSCs) with self-renew ability. Through the transplantation of $VNN2^+$ or $VNN2^-$ HSCs cells into NSG mice, they found that only the $VNN2^+$ HSCs had the capacity to engraft and self-renew after transplantation. They also observed that VNN2 colocalizes with ITGAM (as already described by Huang and colleagues[20]) and confirmed that both are necessary for the maintenance of HSCs properties. Moreover, when they knocked down VNN2 or ITGAM, transduced HSCs were not able to engraft the bone marrow after transplantation into NSG mice suggesting that these two proteins work together and are required for maintaining HSCs function. Thus, if we could confirm a similar role of VNN2 on leukemia cells, VNN2 could potential be a therapeutic target in the future. Scrutinizing the functional role of VNN2 in IR and HR ALL is ongoing.

6. Conclusion

In conclusion, my Master Thesis contributed to the development of a *VNN2* knockout *TCF3-HLF* cell line. These preliminary data constitute the basis for further *in vivo* investigation of the functional role of VNN2 in SR and HR ALL.

A different monoclonal antibody for VNN2 was also validated through an overexpression system as well as in *TCF3-HLF* positive ALL patients. These two experiments have contributed as supplementary figures in a paper that will be submitted in the future:

Vanin-2 (GPI-80) identifies aggressive subtypes of childhood acute lymphoblastic leukemia

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Abstract

The glycophosphatidylinositol anchored surface protein Vanin-2 (VNN2, GPI-80), which has been implicated in leukocyte adherence and migration, identifies human fetal liver hematopoietic stem/ progenitor cells (HSPCs) with self-renewal ability and is required for their hematopoietic function. Comparing the cell surface glycoproteome of 19 acute lymphoblastic leukemia (ALL) samples, we identified VNN2 as a unique feature in patients with a very high risk of relapse by minimal residual disease. In a retrospective analysis of

663 patients on the ALL-BFM-2000 treatment protocol high VNN2 transcript levels were associated with decreased event free survival. We show in a subset of this cohort that VNN2 detection by flow cytometry may serve as a prognostic marker instead. Furthermore all of 12 *TCF3-HLF*-positive ALL, which defines a currently incurable ALL subtype, were strongly surface VNN2, providing a simple procedure to preselect samples for specific diagnostic testing. VNN2 expression was not associated with other cytogenetic and copy number abnormality. Antibody interference with VNN2 resulted in delayed homing of ALL to the bone marrow of immunodeficient mice, suggesting a potential role of VNN2 in leukemia trafficking. Thus surface VNN2 expression identifies *TCF3-HLF*-positive ALL as well as subset of ALL with unfavorable biology that cannot be defined by other diagnostic features and warrants prospective clinical investigation.

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